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CONNECTIVE TISSUE ACTIVATION. XVII. RADIOIMMUNOASSAY OF A HUMAN PLATELET DERIVED CONNECTIVE TISSUE ACTIVATING PEPTIDE (CTAP-III) AND SPECIFICITIES OF ANTI-CTAP-III SERA

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Summary

The platelet-derived connective tissue activating peptide (CTAP-III) has been shown to be an important factor stimulating the metabolism and proliferation of human connective tissue cell strains, including synovial tissue cells. The quantities of CTAP-III affecting the cellular changes and the amounts in various biologic fluids and tissues are small. The objectives of this study were to develop a radioimmunoassay (RIA) for CTAP-III and to ascertain the specificities of the anti-CTAP-III sera reagents. The antisera were shown not to cross-react with a number of polypeptide hormones. However, two other platelet proteins, β-thromboglobulin and low affinity platelet factor-4, competed equally as well as CTAP-III for anti-CTAP-III antibodies in the RIA system. Thus, the three platelet proteins are similar or identical with respect to those portions of the molecules constituting the reactive antigenic determinants. The levels of material in normal human platelet-free plasma that inhibited anti-CTAP-III—^{125}I-CTAP-III complex formation were determined to be 34 ± 13 (S.D.) ng/ml.

Introduction

The connective tissue activating peptide-III (CTAP-III) is a platelet derived polypeptide which causes increased glucose uptake, glycosaminoglycan synthesis, sulfate incorporation and lactate output in a number of fibroblast cell strains [1—4]. CTAP-III has also been shown to stimulate [^{3}H]thymidine incor-

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poration in cultured dermal and synovial fibroblasts, in chondrocytes and in an endothelial cell strain [1,3]. It is a low molecular weight protein (by amino acid analysis) and is cationic as determined by ion-exchange chromatography [2], isoelectric focusing [3] and immunoelectrophoresis [5].

CTAP-III has been found to be antigenically similar to two other platelet-derived proteins, β-thromboglobulin (βTG) and low affinity platelet factor 4 (LA-PF$_4$) by double immunodiffusion analyses [3]. LA-PF$_4$ has been shown to be very similar to CTAP-III in amino acid composition, molecular weight and biological activities, whereas βTG has a different molecular weight (8851 daltons per subunit) and does not demonstrate the biological activities of CTAP-III [3].

The studies presented here were begun in order to develop a sensitive immunoassay for the CTAP-III protein. The details of the radioimmunoassay (RIA) are presented together with data on the cross-reactivities of anti-CTAP-III sera and the levels of CTAP-III in normal human plasma.

Materials and methods

CTAP-III was purified to apparent homogeneity by previously published methods [2,3]. Briefly, outdated platelets were extracted with 10 vol of 5% 1.25 mol/l HCl: 95% ethanol and CTAP-III material in the supernatant precipitated by the addition of 3 vol of acetone. The precipitate was dissolved in and dialyzed against 1.0 mol/l acetic acid containing 3.6% NaCl followed by dialysis against 0.5 mol/l phosphate buffer, pH 7.0, containing 0.15 mol/l NaCl. Precipitates formed during dialysis were removed by centrifugation. The supernatant was chromatographed on Sephacryl S-200 and material eluting in the cytochrome C position were further purified on a CM-Sephadex C-50 ion-exchange resin equilibrated with 0.1 mol/l phosphate buffer, pH 6.0. CTAP-III was eluted from the CM-Sephadex using 0.25 mol/l NaCl in the phosphate buffer. The CTAP-III preparations used for immunizations and in the RIA experiments were homogeneous by the criteria of giving single protein bands following polyacrylamide gel electrophoresis in both sodium dodecyl sulfate and acid-urea systems.

Anti-CTAP-III sera were produced in rabbits as previously described [5]. The rabbits were given multiple intramuscular, subcutaneous and intraperitoneal injections of the purified CTAP-III emulsified in complete or incomplete Freund's adjuvant on an approximately biweekly schedule. High titer and apparent high affinity anti-CTAP-III sera were obtained after the 4th immunization and the high titers of the antisera were maintained for over a year following booster immunizations. The anti-CTAP-III sera gave strong precipitin bands in double diffusion analyses against all human platelet extracts and human sera tested but were unreactive with platelet-poor plasma. The antisera were also unreactive in double diffusion analyses with the polypeptides and growth factors listed below but gave single precipitin bands with βTG and LA-PF$_4$ which fused with the CTAP-III precipitin band [3,5].

CTAP-III was radiolabeled by a modification of the method of Hunter and Greenwood [6] with all steps being carried out at 0—4°C. Approximately 5 μg of lyophilized CTAP-III was dissolved in 90 μl of 0.67 mol/l NaPO$_4$ buffer, pH

7.0, and 1 mCi [^{125}I]Na (Amersham Corp., Arlington Heights, IL) was added. Chloramine-T solution, 2 mg/ml, and sodium metabisulfite, 3 mg/ml, were made fresh in cold distilled water. Ten μl of chloramine-T solution was added followed 10 s later by 10 μl of sodium metabisulfite plus 100 μl each of 1 mol/l KI and 1% human serum albumin (HSA). The ^{125}I-CTAP-III was separated from free ^{125}I on a 8.5 ml Sephadex G-25 column equilibrated and eluted with 0.1% HSA in 0.01 mol/l sodium phosphate buffer, pH 7.2, containing 0.154 NaCl (phosphate buffered saline, PBS) and 0.01% NaN$_3$.

Immunoreactive ^{125}I-CTAP-III was separated from non-reactive radiolabeled protein by reisolating it with an insolubilized anti-CTAP-III antibody using the method of Anderson et al. [7]. Briefly the procedure was as follows. One vol. of a saturated solution of ammonium sulfate was added to 2 vol of rabbit anti-CTAP-III serum, and the precipitate obtained was washed and concentrated to 50 mg/ml of protein using an Amicon Centriflo cone (Amicon Corp., Lexington, MA, mol. wt. exclusion of 25 000). Glutaraldehyde (25%) was added to a final concentration of 1% and the cross-linked insoluble proteins were washed with PBS. ^{125}I-CTAP-III from the Sephadex G-25 void volume was added to the glutaraldehyde cross-linked immunoadsorbent, and the mixture rotated for 2 h at 22°C and overnight at 4°C. The mixture was centrifuged at 1000 × g at 4°C for 15 min and the supernatant discarded. The pellet was washed 3 times with PBS. One ml of 0.1 mol/l glycine-HCl, pH 2.2, was then used to elute ^{125}I-CTAP-III from the immunoabsorbent. The glycine-HCl elution was repeated twice, the three supernatants combined, 1.5 ml of 1% HSA added, and the eluates dialyzed against PBS containing 0.5% NaN$_3$ overnight at 4°C in Spectrapor tubing (Spectrum Medical Industries Inc., Los Angeles, CA, mol. wt. exclusion of 2000).

Titrations of antisera were initially attempted using the Farr technique [8] for processing of RIA mixtures. It was found that 60—80% of the ^{125}I-CTAP-III precipitated at 50% ammonium sulfate concentration with both non-immunized rabbit sera or with the anti-CTAP-III sera, even though purified CTAP-III was soluble at the same ammonium sulfate concentration. Therefore, the ammonium sulfate method for separating free ^{125}I-CTAP-III from antibody-bound ^{125}I-CTAP-III was not workable and the second antibody precipitation method [9] was utilized for precipitation of anti-CTAP-III antibody—^{125}I-CTAP-III complexes in the RIA. Appropriate dilutions of antisera were made in normal rabbit serum diluted 1 : 300 in 0.1% HSA-PBS containing 0.5 mol/l NaCl and 0.01% NaN$_3$. Three hundred μl of the diluted antiserum, unlabeled CTAP-III standards or other samples and 10—20 μl of ^{125}I-CTAP-III (1—2 ng) were added to 12 mm × 75 mm polystyrene or glass tubes and the tubes incubated for 1 h at 22°C. Sixty microliters of second antibody, goat anti-rabbit IgG (Miles-Yeda, Elkhart, IN), was then added to each tube and incubation was continued at 37°C for 2 h followed by 30 min at 4°C. The tubes were centrifuged at 1500 × g for 30 min at 4°C and supernatants removed. The pellets were counted and compared to the total counts added. The antiserum titers were defined as the dilutions of antisera binding 50% of the total ^{125}I-CTAP-III added to the incubation mixtures. Non-specific binding of ^{125}I-CTAP-III to the precipitates was determined using assay mixtures minus the anti-CTAP-III serum. All samples were run in duplicate or triplicate.

Several hormone polypeptides and growth factors were available from commercial and other sources for testing of inhibitory activities in the CTAP-III RIA. These experiments were performed to ascertain cross-reactivities with the anti-CTAP-III sera which would indicate possible structural homologies like those shown between insulin and nerve growth factor [10]. Furthermore, because βTG and LA-PF$_4$ react with anti-CTAP-III sera in double immunodiffusions analysis [3,5], the extent of cross-reactivities in the CTAP-III RIA system were also determined. The various growth factors and hormones used were: bovine insulin and parathyroid hormone (Eli Lilly Co., Indianapolis, IN), porcine glucagon (Lilly), human corticotropin and bovine thyrotropin (Armour Pharmaceuticals, Kankakee, IL), human chorionic gonadotropin (Ayerst Co., Chicago, IL), human gastrin I and bradykinin (Chemical Dynamics), synthetic somatostatin (Sigma Chemical Co., St. Louis, MO), bovine fibroblast growth factor (FGF) and mouse epidermal growth factor (Collaborative Research, Waltham, MA), synthetic porcine luteinizing release hormone (Beckman Instruments, Palo Alto, CA), human prolactin (Calbiochem, La Jolla, CA), mouse nerve growth factor (obtained from Dr. J. Tomita), platelet factor-4 (PF4, obtained from Dr. M. Ginsberg), low-affinity platelet factor-4 (LA-PF$_4$, supplied by Dr. S. Niewiarowski), and β-thromboglobulin (βTG, supplied by Dr. D. Pepper).

Platelet-free plasma samples were collected from healthy volunteers. Venous blood was drawn into plastic syringes and gently transferred into chilled tubes containing EDTA and theophylline as anti-platelet release anticoagulants (Amersham Corp.). After centrifugation at $2500 \times g$ for 30 min at $4°C$ the supernatant was removed and frozen ($-70°C$) until assayed.

A number of animal sera were tested for their ability to inhibit anti-CTAP-III$-$[125]I-CTAP-III complex formation. These included human, rhesus monkey, horse, bovine, fetal calf, lamb, goat, mouse, rat, chicken and porcine sera. Blood was drawn into plastic syringes and allowed to clot in glass tubes for 1 h at $22°C$. Serum was removed and centrifuged at $2500 \times g$ for 30 min and stored at $-70°C$ until used.

Results

The chloramine-T radioiodination procedure incorporated between 9.8 and 21% of the available ^{125}I into the CTAP-III. Specific activities were calculated to range from 4.3 to 12.8 μCi/μg CTAP-III. When the ^{125}I-CTAP-III preparations reisolated from the anti-CTAP-III immunoadsorbent were tested in the double antibody RIA system the radiolabeled antigens were 80—90% bound by antiserum and only 20% or less of the total counts were non-specifically precipitated when using preimmune or normal rabbit serum.

Titers of a number of rabbit anti-CTAP-III sera were determined. Anti-CTAP-III serum (week 28) was drawn 21 days after the 11th immunization and titered at a 1 : 60 000 dilution. Titers of weeks 32 and 12 sera drawn 21 and 7 days after the 12th and 5th immunizations, respectively, had titers of 1 : 60 000 and 1 : 17 000. The high titer antisera suggested that high affinity antibodies may have been produced in the rabbit. A second rabbit immunized with CTAP-III showed similar high titer antisera.

The curves for the inhibition of ^{125}I-CTAP-III—anti-CTAP-III antibody complex formation using CTAP-III, βTG and fibroblast growth factor (FGF) are shown in Fig. 1. Inhibition with unlabeled CTAP-III was seen at 0.5 ng and near-complete inhibition obtained with 10 ng. The same percent inhibition was obtained on a weight basis using LA-PF$_4$ (data not shown). Also on a weight basis, βTG inhibited the complex formation at lower amounts than when CTAP-III was used, and 95% inhibition was obtained with 10 ng of βTG in the incubation mixture. When the same data were plotted on a molar basis of the inhibitors (Fig. 2) using molecular weights of 9295 daltons for CTAP-III [3] and 8851 daltons for βTG [11], the two inhibition curves were equivalent. FGF also competed for anti-CTAP-III—[125]I-CTAP-III complex formation but much higher amounts were required, 1000 ng giving 77% inhibition. All other peptides and protein hormones listed in the Materials section showed no inhibition with addition of 5 μg amounts to the RIA mixtures.

The equivalent molar inhibitions of CTAP-III and βTG in the RIA system suggested that those antibodies binding to ^{125}I-CTAP-III under the conditions of the assay (1 : 40 000 dilution of antiserum) recognized similar or identical antigenic determinants of the two proteins. Furthermore, the finding that βTG completely inhibited the complex formation indicated that there was no antibody specificity reactive with antigenic determinants unique to CTAP-III. In order to increase the probability of detecting possible antibodies specific for CTAP-III the RIA was conducted with the same antiserum diluted 1 : 300. As seen in Fig. 2, both βTG and CTAP-III gave equivalent inhibition values throughout the curve and both inhibited anti-CTAP-III—[125]I-CTAP-III complex formation nearly completely. A series of double immunodiffusion analyses were also performed placing CTAP-III and βTG in adjacent antigen wells and allowing diffusion against anti-CTAP-III sera of both immunized rabbits from the early through the last weeks of immunization. In all cases single precipitin lines were obtained with both βTG and CTAP-III which fused with no detectable spurring.
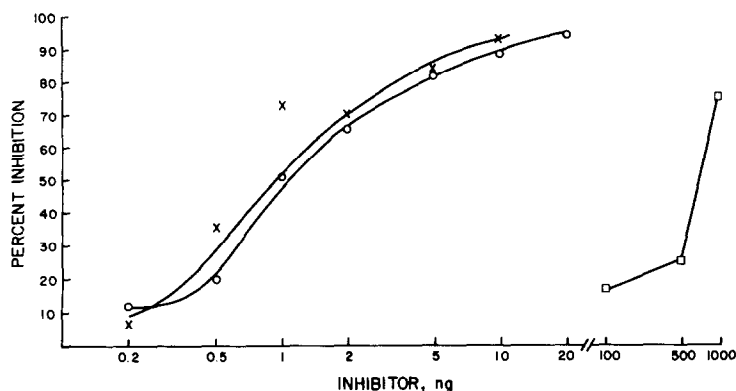

Fig. 1. Inhibition of anti-CTAP-III—[125]I-CTAP-III complex formation by CTAP-III, βTG and fibroblast growth factor. The RIA procedure was performed as described in the text. The anti-CTAP-III serum was used at a dilution of 1 : 60 000. Each point represents the mean of duplicate or triplicate determinations: (●), CTAP-III; (X), βTG; (□), fibroblast growth factor.
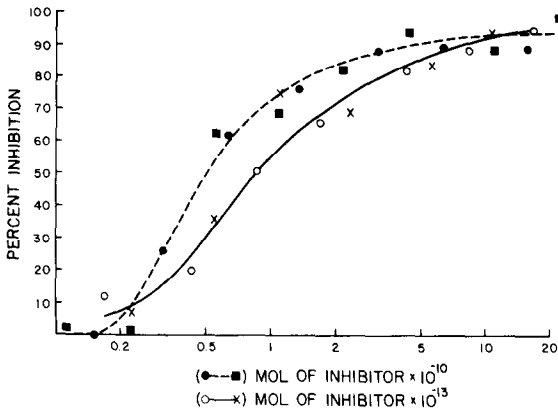
Fig. 2. Inhibition curves for CTAP-III and βTG plotted as the molar quantities of the inhibitors added to the incubation mixtures. The curves are drawn for anti-CTAP-III serum used at a dilution of 1 : 300: (●------●), CTAP-III; (■------■), βTG.

The CTAP-III antigen was quantitated in 37 platelet-free human plasma specimens from healthy adults and the values ranged from 14 to 74 ng/ml. The calculated mean ± S.D. was 34 ± 13 ng/ml. In order to confirm the accuracy of the RIA system when measuring plasma CTAP-III antigen, it was necessary to evaluate the effect of plasma on the assay. Samples of platelet-free plasma (20 μl per assay mixture) produced no consistent change in the non-specific binding of ^{125}I-CTAP-III in the assay procedure. This suggested that human plasma lacks a specific CTAP-binding protein. Also, when known amounts of CTAP-III were added to a constant amount (15 μl) of plasma (containing 0.52 ng of antigenically reactive material), the inhibition curve was coincident with
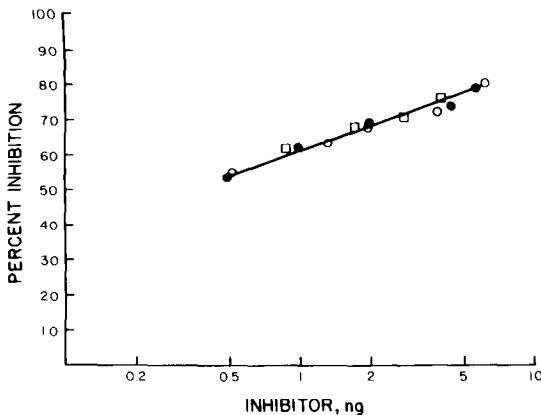

Fig. 3. Lack of interference by plasma on anti-CTAP-III—[125]I-CTAP-III complex formation. A standard inhibition curve with CTAP-III (●) without plasma is compared to an inhibition curve of different amounts of CTAP-III added to 15 μl of plasma (○). The data point on the left (equal to 0.52 ng CTAP-III) is the 15 μl aliquot of the plasma sample without addition of purified CTAP-III. A plasma sample containing a relatively high amount (4.2 ng) of CTAP-III reactive material was diluted 1.3-, 2- and 4-fold and each dilution assayed (□). The point on the far right represents the 20 μl aliquot of undiluted plasma.

the CTAP-III inhibition curve without plasma added (Fig. 3). Furthermore, a plasma sample containing a relatively large amount of anti-CTAP-III reactive material could be diluted serially to give a coincident inhibition curve to that of CTAP-III (Fig. 3).

A sample of human serum and fresh specimens of other animal sera (listed under "Materials") were also tested for inhibition of the anti-CTAP-III—[125]I-CTAP-III complex formation. The human serum contained approximately 10 μg/ml of CTAP-III reactive material. The rhesus monkey serum was also inhibitory in the RIA system whereas all other animal sera did not inhibit the complex formation.

Discussion

The experimental details for the quantitation of CTAP-III by RIA have been presented. High non-specific binding of ^{125}I-CTAP-III was observed with protein precipitated from serum using the 50% concentration of ammonium sulfate. Such binding may in part result from the cationic nature of the molecule or may be due to another serum protein which binds CTAP-III relatively specifically. Thus, high salt concentration (0.5 mol/l NaCl) was needed to reduce this non-specific binding of ^{125}I-CTAP-III in the precipitation of antibody-antigen complex step. It was also necessary to reisolate the ^{125}I-CTAP-III from an immunoadsorbent of glutaraldehyde-insolubilized anti-CTAP-III antibodies. Either certain iodination sites may alter the antigenic determinants of the molecule or the iodination conditions resulted in substantial denaturation. The disulfide bonds of CTAP-III are known to be important for the expression of biologic activity [2] and their reduction in the iodination procedure may also have altered the antigenic activity.

Radioimmunoassays with several of the anti-CTAP-III sera diluted in the range of 1 : 20 000 to 1 : 60 000 gave comparable inhibition curves. The RIA was very sensitive with a workable range of determining between 0.5 and 10 ng of CTAP-III reactive material, the other components present in the plasma samples did not affect the assay system.

CTAP-III, LA-PF$_4$ and βTG all gave essentially identical curves of inhibition of anti-CTAP-III—[125]I-CTAP-III complex formation when plotted on a molar basis of the inhibitors. LA-PF$_4$ was shown previously to have the same biologic properties of CTAP-III (3) and the present results confirm the probable identity of the two substances [3,13]. βTG does not have the biologic properties of CTAP-III and is slightly smaller in molecular weight [3,11,12,14], yet the two molecules are identical in antigenic reactivity with the anti-CTAP-III sera. It is likely that the two molecules are related, with βTG being derived from CTAP-III through proteolytic action [14]. Therefore, the immunoassay of CTAP-III antigen in biologic samples would measure both βTG and CTAP-III. Also, we have shown that anti-βTG antiserum—[125]I-CTAP-III complex formation is inhibited equivalently on a molar basis by both βTG and CTAP-III. Thus, RIAs quantitating βTG also include the amount of CTAP-III present in the sample assayed. The relative content of these two platelet factors in plasma and other biologic specimens have not as yet been determined.

The additional sequence of CTAP-III compared to βTG raises the question of

whether anti-CTAP-III sera may have an antibody population binding to an antigenic determinant of that sequence. No evidence was found to suggest that anti-CTAP-III specific antibodies were present. In the RIA system at low antiserum dilutions (1 : 300) no differentiation of βTG and CTAP-III inhibition curves was made. Also, no spurring of precipitin band formation was obtained in double immunodiffusion analyses of the large number of anti-CTAP-III sera tested. Reasons for the lack of specific anti-CTAP-III antibodies may be the following. The CTAP-III may undergo proteolytic cleavage to βTG or smaller fragments at the immunization sites, or, the additional sequences of CTAP-III may be highly conserved and the rabbits may contain identical or very similar sequences and thus not recognize that portion of the molecule as sufficiently foreign to be immunogenic. Studies are in progress to prepare reagents which will differentiate CTAP-III from βTG.

Of all the other substances tested for possible inhibitory capabilities in the anti-CTAP-III RIA, only the bovine FGF was active at about a 100-fold greater weight ratio. Both CTAP-III and FGF exhibit many similar biologic properties [3] although their amino acid compositions are different. FGF may have an antigenic determinant amino acid sequence(s) similar to, but poorly crossreactive with that of CTAP-III. Alternatively, the FGF preparations may contain a CTAP-III-like substance as a contaminant.

The levels of CTAP-III reactive components in human plasma were determined to have a mean value of 34 ng/ml for the 37 samples tested. This is similar to the values of βTG in plasma of 30.5 ng/ml and 25.83 ng/ml obtained by Ludlam and Anderton [12] and Brown et al. [15], respectively. As noted by Dawes et al. [16] it is not yet clear how much of the βTG (and CTAP-III) found in platelet-free plasma results from platelet release during venipuncture and sample preparation. The human serum concentration of the CTAP-III reactive component was at least 10 μg/ml, 250-fold greater than the human plasma value and representing the CTAP-III-βTG components of the platelet released in the clotting process. It is interesting that other animal sera, except for the rhesus monkey serum, contained little or no reactivity in the RIA system. Thus, assuming that other species of animal have similar molecules with CTAP functions, the anti-CTAP-III sera are very specific for the human and monkey molecules.

Acknowledgements

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